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**The role of Arabidopsis ABA receptors from the PYR/PYL/RCAR family in stomatal acclimation and closure signal integration.**

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## 34 **Abstract**

35 Stomata are microscopic pores found on the surfaces of leaves which act to control  
36 CO<sub>2</sub> uptake and water loss. By integrating information derived from endogenous  
37 signals with cues from the surrounding environment the guard cells, that surround the  
38 pore, ‘set’ stomatal aperture to suit the prevailing conditions. Much research has  
39 concentrated on understanding the rapid intracellular changes that result in  
40 immediate changes to stomatal aperture. In this study we look instead at how  
41 stomata acclimate to longer timescale variations in their environment. We show that  
42 the closure-inducing signals ABA, increased CO<sub>2</sub>, decreased relative air humidity  
43 (RH), and darkness each access a unique gene network made up of clusters (or  
44 modules) of common cellular processes. However, within these some gene clusters  
45 are shared among all four stimuli. All stimuli modulate the expression of members of  
46 the PYR/PYL/RCAR family of ABA receptors, however these are modulated  
47 differentially in a stimulus-specific manner. Of the six members of the  
48 PYR/PYL/RCAR family expressed in guard cells, PYL2 is sufficient for guard cell  
49 ABA-induced responses. Whereas, in the responses to CO<sub>2</sub> PYL4 and PYL5 are

50 essential. Overall our work shows the importance of ABA as a central regulator and  
51 integrator of long-term changes in stomatal behaviour, including sensitivity, elicited  
52 by external signals. Understanding this architecture may aid breeding of crops with  
53 improved water and nutrient efficiency.

## 54 **Introduction**

55 Stomata are pores on the leaf surface bounded by two guard cells. Their role is to  
56 optimize gas exchange in changing environmental conditions<sup>1-3</sup>. In the light, stomata  
57 open, allowing CO<sub>2</sub> to enter the leaf for photosynthesis. In contrast, darkness, high  
58 concentrations of CO<sub>2</sub>, reductions in RH which is equivalent to an increase in the  
59 water vapor pressure deficit (VPD) and exposure to the hormone abscisic acid (ABA)  
60 promote stomatal closure<sup>4-8</sup>. This is a rapid process typically lasting from 20 – 40  
61 minutes. Reductions in stomatal aperture are the result of turgor and volume  
62 reduction in the respective guard cell pair. The key player in ABA- and CO<sub>2</sub>-  
63 dependent guard cell deflation is the guard cell anion channel SLAC1 together with  
64 the SLAC1-activating protein kinase OST1<sup>9</sup>. Under low, sub-threshold concentrations  
65 of ABA, the protein phosphatase ABI1 keeps the guard cell anion channel kinases  
66 dephosphorylated and SLAC1 inactive<sup>10</sup>. When the concentration of ABA increases it  
67 binds to receptors of the PYL/PYR/RCAR family<sup>11-13</sup>. ABA-receptor interaction  
68 prevents the ABI1-mediated inactivation of the SLAC1 kinase. This results in anion  
69 channel opening, which in turn brings about depolarization of the plasma membrane.  
70 The change in membrane potential results in the release of osmotically active K<sup>+</sup> and  
71 anions. This is followed by osmotic water release causing guard cells deflation and  
72 stomatal closure<sup>4,9,14,15</sup>.

73

74 Over the last 40 years most investigations have concentrated on identifying and  
75 understanding how individual guard cell proteins and intracellular second  
76 messengers interact to bring about these rapid changes in stomatal aperture. In  
77 contrast, we know much less about the processes that underlie stomatal acclimation  
78 to longer term environmental changes<sup>16</sup>. These involve alterations in gene  
79 expression, which result in changes in stomatal development and function. In the  
80 former category it is recognized that plants acclimate to increases in the  
81 concentration of atmospheric carbon dioxide and light intensity by decreasing the  
82 number or size of stomata that develop on the surface of plant leaves<sup>17-21</sup>. Reducing  
83 stomatal density is known to increase plant water use efficiency<sup>22</sup>. Stomata also  
84 acclimate to changing environments by altering their sensitivity to ABA. In  
85 *Arabidopsis* this happens during development with the youngest guard cells being  
86 ABA insensitive<sup>23</sup>. Guard cell sensitivity to ABA is modulated by water stress or  
87 exposure to increased VPD<sup>23,24</sup>. In these cases, “switching on” ABA responsiveness  
88 provides the plant with the ability to control its water relations.

89 Understanding how stomata acclimate to changes in their environment has clear  
90 relevance to the global challenge of producing crops that are more resilient to  
91 environment change. Here we identify changes in guard cell gene expression that  
92 are likely to contribute to the mechanisms that allow stomatal aperture responses to  
93 adapt in the longer term to changes in closure-inducing environmental signals. We  
94 reveal that these responses are characterised by the expression of both stimulus-  
95 specific suites of genes and core genetic modules that are regulated by all closure-  
96 inducing stimuli. We also suggest that part of stomatal acclimation to all these signals  
97 is achieved by controlling the sensitivity of the guard cell to ABA. This is achieved by  
98 differentially regulating the expressions of members of the PYR/PYL/RCAR family of

ABA receptors. Moreover, we show, in the cases of ABA and elevated CO<sub>2</sub>, that response specificity is achieved by these signals accessing different members of the PYR/PYL/RCAR family of receptors.

#### *Stimulus-specific modulation of guard cell gene expression.*

To investigate the effects of ABA, low air humidity / increased VPD, darkness, and elevated CO<sub>2</sub> on guard cell gene expression we used a microarray-based approach. We reanalysed two microarray data sets (ABA and RH / VPD treatment) from a previous study<sup>6</sup> and combined them with two new microarray studies where we used darkness and high CO<sub>2</sub> as stomatal closing signals (for detailed analysis of the differentially expressed genes see Supplementary Table 1a). We first analysed the resulting data set using classical pathway analyses and this revealed that each closure-inducing signal addressed a defined, unique cluster of gene pathways (Fig. 1, Supplementary Fig. 1a and Supplementary Information 1). We also found that gene clusters are shared among two, three or all four stimuli. For example, the shared clusters for ABA and lowered RH (equivalent to increased VPD) are dominated by the ABA response (Supplementary Fig. 1b) and the LEA pathways and are up-regulated by both treatments. To gain further information about the interrelationship of these pathways we analysed the co-expression patterns of the genes from each pathway using the public ATTED II database<sup>25</sup>. This analysis revealed that both pathways are highly interconnected (Supplementary Fig. 1c to e). Notably, *RAB18*, *DAA1* and *MYB74*, which we had previously identified as being core to RH (VPD) and ABA induced guard cell responses<sup>26</sup> were co-expressed with

several other genes common to both responses, such as the dehydrins and members of the *HVA22* family (Supplementary Fig. 1c to e).

The shared gene cluster of darkness and low RH (high VPD) is dominated by abiotic stress and glucosinolate pathway genes. Both clusters contain flavin monooxygenases/glucosinolate oxygenases (*FMOGS*), which are responsible for the last step of side chain modification of aliphatic glucosinolates. *FMOGS* are involved in biotic and abiotic stress responses in general and most of them are induced by ABA<sup>27</sup>. The shared cluster for darkness/CO<sub>2</sub>, ABA/darkness, ABA/CO<sub>2</sub> and RH/CO<sub>2</sub> are dominated by more general metabolic pathways, receptor kinases, cell wall degradation, major carbohydrate metabolism, glucosidases and transport (Supplementary Fig. 1a). To directly model these complex interconnections, we performed an integrative network analysis based on a rigorous mathematical approach (see Material and Methods).

Specifically, we incorporated the set of protein-protein interactions from the STRING database, which primarily represents physical interactions between proteins, into our analysis. By integrating these data we add a new dimension into the expression data. In contrast to the co-expression network, this allows the detection of functional signalling links at the post-translational protein level. We analysed the network on the basis of the measured expression levels into functionally coherent modules. These can be regarded as the building blocks of cellular processes, such as basic signalling cascades. The resulting optimal solutions represent maximally responsive network modules with respect to the specific experimental stimuli.

This approach is hypothesis free and allows an unbiased exploration of stimulus-triggered network responses, and in particular can integrate novel, hitherto unknown,

genes and connections into a pathway. Based on this algorithm we were able to expose clusters and connections, which cannot easily be detected by classical methods. This revealed stimulus-specific gene modules responsive to different stomata closing signals (Fig. 2a-d) (for details see Supplementary Information 2, Supplementary Table 1b and Supplementary Fig. 2a-d).

#### *Closure-inducing signals differentially modulate PYR/PYL/RCAR receptor expression.*

Another feature to emerge from our investigations was that the different closure-inducing signals led to differential expression of ABA receptor family members in guard cells. At the level of the whole plant and at the tissue level it has been documented that ABA application regulates the expression of certain PYR/PYL/RCAR receptors<sup>13,28</sup>. In our investigations we found that six guard-cell-localized *PYR/PYL/RCARs* were affected (Fig. 2e). In the case of ABA, three receptors (*PYL2*, 4, and 5) were down-regulated while darkness up-regulated *PYR1*. Exposure to dry air (increased VPD) resulted in the downregulation of *PYR1*, *PYL2*, 4, and 8. In high CO<sub>2</sub>, *PYL8* is up-regulated while *PYL2* is down-regulated. These data suggest that ABA and receptors of the *PYR/PYL/RCAR* family are involved in the response to all these closure-inducing stimuli. One possible interpretation of these results is that, in the long term, acclimation to these signals is achieved through modulating the transcript abundance of individual *PYR/PYL/RCAR* family members. The net result of this would be to fine-tune guard cell sensitivity to ABA. In this context it is interesting that we found that transcript abundance for these receptors was differentially sensitive to ABA concentration. Specifically, we observed that 250 nM ABA induced a reduction in *PYL4* abundance while changes in the



abundance of *PYL2*, 4, 5, 8, and *PYR1* were only observed on treatment with 10X this concentration of ABA (2.5uM). In this context, it should be mentioned that the products of ABA metabolism might interact with ABA receptors. This is indeed the case for phaseic acid that is capable of binding to *PYL2*<sup>29</sup>. It is therefore possible that these metabolic products could also regulate transcriptional changes of the receptor genes. We next investigated whether other guard cell ABA-regulated genes were, like the *PYR/PYL* genes, differentially regulated in a concentration-dependent manner. As is apparent from the data in Table 1 and Supplementary Fig. 3 (see for details and statistics), *ABR*, *HAI1* and *MYB60* responded to ABA stimulation in a concentration dependent manner. Together, these observations suggest a framework in which ABA regulates specific receptor family member abundance in a concentration dependent manner. This would potentially provide a high degree of control over receptor and response sensitivity.

#### *Differential guard cell gene expression regulated by PYR/PYL family members.*

We next decided to investigate the possibility that different members of the *PYR/PYL/RCAR* receptor family differentially regulate gene expression. For this we used Arabidopsis *PYR/PYL/RCAR* receptor mutants. The Arabidopsis genome contains 14 *PYR/PYL/RCARs* and previous studies have shown that generation of quadruple, pentuple and sextuple mutants is required to obtain robust ABA-insensitive phenotypes<sup>12,28,30</sup>. However, both biochemical analyses of different receptor-phosphatase complexes and receptor gene expression patterns suggest that the function of ABA receptors is not completely redundant<sup>28,31,32</sup>. Analysis of the single *pyl8* mutant, for example, has revealed a non-redundant role of *PYL8* in root sensitivity to ABA<sup>33,34</sup>, while the *PYL9/RCAR1* receptor specifically regulates the

protein phosphatase AHG1<sup>32</sup> and promotes leaf senescence<sup>35</sup>. Additionally, a non-redundant function of the dimeric receptor BdPYL1 has been reported in *Brachypodium*<sup>36</sup>. Functional diversification follows the evolutionary expansion of a gene family, therefore we investigated the role of different ABA receptors in guard cells.

Our transcriptomic data confirmed that guard cells express the six ABA receptors *PYR1*, *PYL1*, 2, 4, 5, and 8 that have previously shown to be relevant for stomatal closure<sup>7,28</sup>. We therefore asked whether, in guard cell ABA signalling, the six different receptors are redundant or if each *PYR/PYL* has individual functions. To answer this question, we used the *pyr/pyl* quintuple mutant known as 12458<sup>28</sup>, where the receptors *PYR1* and *PYL2*, 4, 5, 8 are knocked out and the 11458<sup>33</sup> loss of function mutant in which *PYR1* and *PYL1*, 4, 5, 8 are knocked out. In addition, we took advantage of the availability of quintuple mutant complementation lines. These lines under the control of the guard cell specific GC1 (At1g22690) promoter<sup>37</sup> express single receptors individually (see details in Supplementary Fig. 4).

To exclude the possibility that the following results were influenced by overexpression of the individual receptors in the complementation lines, we measured the expression levels of *PYR1*, *PYL1*, *PYL2*, *PYL4*, *PYL5* and *PYL8* in guard cells of wildtype, both quintuple mutants and the complementation lines in the 12458 background (Supplementary Fig. 5a-f). These results showed that neither *PYL1* (present in 12458) nor *PYL2* (present in 11458) were up-regulated in the quintuple mutants to compensate for the lack of the ABA receptors that were knocked out. Furthermore, the complementation lines showed wildtype-like

expression of the respective receptors (maximally varying about 2-3fold). To confirm that ABA receptor RNA abundance is mirrored on protein level, we selected PYL2 as test case for a mass-spectrometry-based proteomic approach, because PYL2 is the most relevant for ABA induced stomatal closure (see below). As expected PYL2 receptor peptides were neither found present in the 12458 mutant nor the associated PYR1, PYL4 und PYL5 complementation lines. In wildtype guard cells and those of the PYL2 complemented 12458 mutant, however, PYL2 peptides were detected at comparable levels (Supplementary Fig. 5g). These findings underline the suitability of the GC1 promoter to drive nearly guard cell physiological ABA receptor expression.

To resolve whether different members of the PYL/PYR receptor family regulated expression of guard cell genes in a differential fashion we concentrated on the ability of ABA to regulate the following known guard cell ABA-responsive genes, *MYB60*, *ABI1*, *ABI2*, *CIPK25* and *KCR2*<sup>6</sup> (Table 2 and Supplementary Fig. 6 for details and statistics). We observed *MYB60* downregulation in wildtype, and 11458 (*PYL2* present) but not in 12458 (*PYL1* present) mutant. Complementation of the latter mutant with *PYR1* or *PYL2* restored the wildtype-phenotype, indicating that ABA-dependent down-regulation of *MYB60* is under control of these receptors. Up-regulation of *ABI2* expression was abolished in 11458 but it was restored by *PYR1* or *PYL1* complementation, indicating that *PYR1* and *PYL1* are sufficient for *ABI2* expression. Likewise, up-regulation of *CIPK25* was abolished in 11458 but restored by *PYL1* or *PYL8*, indicating they are sufficient for *CIPK25* expression. A complex regulation of *KCR2* expression seems to occur since ABA-induced upregulation was abolished in 11458 (*PYL2* present) and could not be restored in complementation lines. Finally, although *RAB18* appeared to be always induced by ABA, the presence

of PYL1 in the 12458-mutant (pval 0,00018) or combined with PYR1 (pval 0,025) led to 12 to 14fold induction (more than threefold of the wt), but when just one of the receptors PYL2, 4, 5 was expressed, this over-induction was suppressed. Since we found no strong differences in the basal levels of the examined genes in the different genetic backgrounds (Supplementary Table 1c), these findings indicate that transcriptional guard cell ABA responses are mediated by individual receptors or distinct *PYR/PYL* combinations.

#### *Loss of PYL2 gene renders stomata ABA insensitive*

The above experiments reveal the role of individual PYR/PYL family ABA receptors in the control of guard cell gene expression. We next turned our attention to investigating whether individual receptor family members have distinct roles to play in the control of stomatal aperture and gas exchange. To do this we first used infrared gas analysis (IRGA)<sup>38</sup>. In this approach we used excised leaves and applied ABA via the petioles to exclude any root-effects on transpiration. Under these conditions wild-type leaves opened their stomata upon illumination and adjusted their aperture to the environmental settings prior to ABA application<sup>39</sup>. We found that in the light, the 12458 quintuple mutant (*PYL1* present) in contrast to the 11458 mutant (*PYL2* present) exhibited a severe open-stomata phenotype that manifested itself in rapid wilting on exposure to light. To overcome this experimental difficulty, we pre-incubated plants in the dark with 25  $\mu$ M ABA for 1h hour before applying the light stimulus. Using this protocol, upon illumination wildtype plants remained in a non-transpiring state (that is ABA inhibited light-induced stomatal opening). In contrast, transpirational water loss of the ABA insensitive 12458 mutant increased over time

(Fig. 3a), whereas the 11458-quintuple mutant, which expressed wild type *PYL2*, behaved almost like wildtype (Fig. 3a). Thus, we tested all available complementation lines in the 12458-background. The 12458-mutant, when complemented by *PYR1*, exhibited reduced transpiration, but the presence of *PYR1*, however, did not fully complement the ABA insensitive phenotype of the quintuple mutant that had lost *PYL2* (Fig. 3b). These data suggest a key role for *PYL2* in the guard cell ABA signalling pathway that leads to stomatal closure. This was confirmed by complementing *PYL2* in the 12458-mutant which resulted in a plant that showed the same ABA suppression of stomatal transpiration as the wildtype (Fig. 3b). In contrast, guard cell expression of *PYL4* and 5 did not complement the wilted 12458-mutant phenotype (Fig. 3c).

In addition to IRGA measurements, which provide data on, in this case, the effects of manipulating gene expression on stomatal transpirational control at the level of the whole leaf, we followed the response of individual stomata in their natural environment of the intact leaves. This was achieved by applying 50 $\mu$ M +/- ABA (corresponding to 25 $\mu$ M biologically active ABA) to single stoma via micropipette-assisted electro-infusion. By introducing ABA directly to the guard cells we aimed to exclude any indirect hormone effects via, for example, the mesophyll cells and, or vasculature on the control of transpiration by stomata. Using this protocol we observed that stomata of wildtype *Arabidopsis* closed within 10 min of exposure to ABA (Fig. 4a and b and Supplementary movies). In contrast stomata of the 12458 mutant (*PYL1* present) did not respond to the hormone and remained open. This mutant was also the only one with significantly wider open stomata under control conditions (Fig. 4c). In the quintuple mutant (12458) complemented by *PYL2* and the

11458 mutant (*PYL2* present), we observed stomatal closure that was identical to WT. We also observed that *PYL5* was not able to complement the ABA insensitive phenotype (Fig. 4a and b and Supplementary movies). This demonstrates that *PYL2* is sufficient to fully rescue guard cell's ABA signalling and fast stomatal closure. To confirm our findings that *PYL2* and partially *PYR1* are able to complement the ABA insensitivity of the 12458 mutant, we repeated the ABA pre-incubation experiment with the *pyl2-1* loss of function mutant (seed strain GT2864)<sup>12</sup> and the corresponding Ler wildtype. Although *PYR1*, *PYL1*, *PYL4*, *PYL5* and *PYL8* are present in this mutant, *pyl2-1* behaved almost like the *PYR1* complementation line in the 12458 background (Supplementary Fig. 7), supporting our hypothesis that *PYL2* and *PYR1* are the key receptors for proper ABA induced stomatal closure.

#### *PYL4 and PYL5 are sufficient for CO<sub>2</sub>-induced stomatal closure*

Since Raschke's research in the 1970s we have known that stomata require ABA to sense elevated CO<sub>2</sub><sup>7,8,40,41</sup>. However, the molecular basis of this interaction has not been clear. We took advantage of the panel of ABA receptor mutants previously described to investigate the basis of the interaction using IRGA. Stomata that had been opened in the light in the presence of 400 ppm CO<sub>2</sub> (ambient) were exposed to 1000 ppm CO<sub>2</sub>. This resulted, as expected in a decrease in transpiration consistent with reduction in stomatal aperture (Fig. 5a). Next, we used the 11458 mutant in which *PYL2* is naturally expressed. We had previously shown that this *PYL2* expressing mutant showed wildtype-like ABA-induced closure response (Fig. 3b). However, in contrast to ABA, CO<sub>2</sub>-induced closure was impaired in this as well as in the 12458 mutant (Fig. 5a). Stomata of complementation lines in the 12458-background (with *PYL1* present) expressing *PYR1* or *PYL2* remained impaired in

their response to high CO<sub>2</sub> as well (Fig. 5b). However, lines that express *PYL4* or *PYL5* in guard cells regained stomatal CO<sub>2</sub> sensitivity similar to wildtype plants (Fig. 5c). These data show that *PYL4* or *PYL5* are sufficient for the induction of closure by increased CO<sub>2</sub>. Interestingly, except the *PYL5* complementation line, all other mutants showed remarkably higher ground levels of transpiration in the light and under ambient CO<sub>2</sub> atmosphere compared to wildtype (Supplementary Fig. 8). Only the *PYL4* complementation line started to reopen their stomata after about 2 h of high CO<sub>2</sub>-treatment. Future studies will investigate whether this behaviour represents a phenomenon associated with adaption (Supplementary Fig. 8).

## Discussion

The primary objective of the work described in this paper has been to explore how stomata adapt to changed environmental conditions. Our focus has not been on changes in stomatal development induced by changes in the environment. Rather, we have concentrated on examining the mechanisms likely to underpin, changes in, for example, the sensitivity of the stomatal aperture response to closure-inducing stimuli. Specifically, when we looked at 4 different closure-inducing stimuli, we found that they regulated a diverse set of intracellular pathways. While there was some overlap there were marked differences and this must reflect specific effects of these signals on guard cell properties. However, markedly, the integrated network analysis (see Supplementary Information 3) revealed that all closure-inducing signals induced changes in the expression of the *PYR/PYL/RCAR* ABA receptor family. This provides a mechanism for all stimuli to modify stomatal function through modulating the sensitivity of the ABA signalling pathway. We investigated the control of guard cell gene expression by ABA in more detail and found that individual genes, including members of the ABA receptor family, responded to ABA in a concentration

dependent manner. This conclusion was strengthened when we found that the expression in guard cells of *PYL4* or *PYL5* was sufficient for CO<sub>2</sub>-induced stomatal closure but was not sufficient to restore the ability of ABA to inhibit light-induced stomatal opening in the 12458 mutant, whereas *PYL2* was sufficient for this response. Our data on the ABA response of the complemented lines agree with a previous publication<sup>42</sup> which showed that *pyr1/pyl1/pyl2/pyl4* quadruple mutant plants did not close their stomata following ABA application and that ABA preincubation could not prevent stomatal opening by light. In contrast, in another study<sup>43</sup> using the same mutant, ABA application to open stomata did also not induce stomatal closure, but ABA preincubation prevented stomatal opening. In the latter paper the authors used guard cells in epidermal peels (obtained by macerating leaves in a waring blender) floating on ABA solutions. Future studies will have to investigate whether and how mechanical stress feeds back on guard cell ABA biology. It should be noted that *pyl2-1* single mutant<sup>12</sup> behaved like the *PYR1* complemented 12458 plants indicating that *PYR1* and *PYL2* are the most important receptors for the fast ABA response of guard cells. However, one might not be able to exclude the possibility, that either *PYL8* or heterodimers involving receptors other than *PYL2*, are also competent to rescue the ABA-insensitive phenotype of the 12458 mutant.

In terms of stomatal CO<sub>2</sub>-responsiveness, a previous report agrees that CO<sub>2</sub>-induced induced stomatal closure is disrupted in ABA-receptor mutant backgrounds<sup>8</sup>. In contrast another paper concludes that “rapid CO<sub>2</sub> signal transduction leading to stomatal closure via an ABA-independent pathway”<sup>44</sup>. How might these radically different conclusions be reconciled? It is of course only possible to speculate. However, as documented earlier<sup>23</sup> stomatal responsiveness to ABA varies from



insensitivity to sensitivity and that this is regulated by relative air humidity (VPD). Modulation of stomatal sensitivity by plant growth conditions such as relative humidity could provide a framework to explain the disparity in the results. The veracity of such a possibility requires further experimentation. Nevertheless, it is worth pointing out that examination of the data in Hsu et al.<sup>44</sup>, like before in Merilo<sup>7</sup>, reveals that the quadruple receptor mutants *pyr1/pyl4/5/8* and hexuple *pyr1/pyl1/2/4/5/8* display reduced CO<sub>2</sub> responsiveness compared with wild type. These data do indicate a role for ABA receptors in the response to CO<sub>2</sub>. This was also the conclusion reached when stomatal conductance in response to CO<sub>2</sub> was analysed in wild type and *pyr1/pyl1/2/4/5/8*<sup>7</sup>. Finally, long-term exposures of the in-gel kinase assays done by Hsu et al.<sup>44</sup>, showed a basal activity of OST1 in guard cells which, according to the authors, is probably necessary for rapid CO<sub>2</sub>-induced stomatal closure. Such basal level of phosphorylation would be very sensitive to phosphatase and PYR/PYL/RCAR activity, which can be modified by nanomolar changes in ABA concentration ( $K_d$  for ternary complexes in the 20-40 nM range<sup>11,13</sup>). However, to return to the current investigation, our data, including its in-depth analysis of receptor mutants, are strongly supportive of a role for ABA receptors, in particular PYL4 and PYL5 in the stomatal response to increased concentrations of CO<sub>2</sub>.

Together our investigations, reveal not only the complexity of the underlying cellular mechanisms responsible for controlling stomatal responses to closure-inducing signals and how this might contribute towards stomatal response acclimation, but they also reveal a plausible mechanism for how stomata might integrate multiple signals. The results here are likely to be of benefit to plant breeders who are

interested in engineering crops to respond to the complex multiple environmental stresses that are likely to be associated with future climates.

## **Material and Methods**

### **Plant Material and Growth Conditions**

*Arabidopsis thaliana* plants were grown in soil that was semi sterilized for 20 minutes at 100 °C and cultivated in climate chambers (Binder KBWF 720; www.binder-world.com) in a 12 h day night rhythm (22/16 °C, 60% RH) and were illuminated with 125  $\mu\text{mol m}^{-2} \text{s}^{-1}$  white light. The gas exchange measurements and the expression studies were performed with 6 to 8 weeks old adult, non-flowering plants.

### **Preparation of the ABA-receptor mutants**

Using Col genomic DNA as template we amplified by PCR the promoter region of At1g22690 located 1716 bp upstream of the start codon, named GC1<sup>37</sup>, using the primers FGC1: 5'-ACCATGGAGTAAAGATTCAGTAACCC and RGC1: 5'-ATCCATGGTATTTCTTGAGTAGTGATTTTGAAG. Next GC1 was cloned in pCR8/GW, excised as a NcoI fragment and cloned in front of either PYR1, PYL2, PYL4, PYL5 or PYL8. The resulting pGC1-PYR/PYL construct was recombined by LR reaction into modified pAlligator2<sup>45</sup>, which has been previously HindIII-EcoRV doubly digested, treated with Klenow and religated to obtain a construct lacking the 35S promoter. Each modified pAlligator2-GC1:PYR/PYL construct was transferred to *Agrobacterium tumefaciens* C58C1 (pGV2260)<sup>46</sup> by electroporation and used to transform 12458 and 11458 mutant plants<sup>28,33</sup> by the floral dip method<sup>47</sup>. T1

transgenic seeds were selected based on seed GFP fluorescence and sowed in soil to obtain the T2 generation. Homozygous T3 progeny was used for further studies. Several *PYL8::12458* complementation lines were also tested but did not grow properly. They showed a severe phenotype and excised leaves wilted immediately. These mutants could not be used in any of our physiological experiments and were thus excluded from our analyses.

### **Infra-Red-Gas-Analyses (IRGA)**

*Stomatal closing assays*: ABA and low air humidity application were performed as described<sup>6</sup>. In brief, ABA solution (50  $\mu$ M +/- ABA) was sprayed to the plants until the surface was fully covered. The low humidity treatment was performed by reducing the RH from 80% to 20% at 22°C what equals a VPD change from 0.529 to 2.116 kPa. High CO<sub>2</sub> (1000 ppm) was applied to plants in airtight boxes after two hours illumination at ambient air to close stomata. For the samples in darkness plants were fully shaded following two hours of illumination. Each closing signal persisted for four hours. To minimize diurnal effects on gene expression, all procedures were started in the morning following two hours after onset of illumination.

*Leaf gas exchange measurements*: Transpiration rates were measured with a custom-made system as described elsewhere<sup>38</sup>. CO<sub>2</sub> response measurements were performed with intact plants at 20°C and 52,5  $\pm$  3% RH (VPD = 1.123 kPa). The soil surface of the pots was tightly covered with plastic foil to avoid water evaporation. After the transpiration rates had stabilized in darkness, plants were illuminated with 125  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> white light. After 1h of illumination the CO<sub>2</sub>-concentration of the air stream was increased from 400ppm to 1000ppm.

The effect of ABA on the light induced transpiration of Arabidopsis was measured with detached leaves and the same conditions as described for the CO<sub>2</sub> measurements. To avoid xylem embolism, the leaves were cut under water. The petioles were directly transferred into 50  $\mu$ M +/- ABA solution and incubated in darkness. After one hour ABA incubation leaves were illuminated with 125  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> white light and the transpirational water loss was recorded.

### **ABA Electro-Infusion**

This method was performed according to<sup>48</sup>. Excised leaves from 5-6-week-old plants were attached to a petri dish using double-sided tape and submerged in a bath solution (1mM KCl, 1mM CaCl<sub>2</sub> 5mM MES/BTP, pH 6). The petri dish was mounted to an upright microscope (Axioskop 2FS, Zeiss, Jena, Germany) and the leaves were left for incubation while being exposed to 125  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> light. Stomata on the leaves abaxial side were visualized with a water immersion objective (Achromat x63/0.9 W, Zeiss) and images were recorded with a camera (CoolSNAP HQ, Visitron Systems, Puchheim, Germany) in 30 sec intervals using VisiView (Visitron Systems) imaging software. Electrodes from borsilicat capillaries (inner/outer diameter = 0.56/1.0 mm; Hilgenberg, Malsfeld, Germany) were pulled on a horizontal laser puller (P2000, Sutter Instrument, Novato, CA, USA) to achieve resistances ranging from 60 to 100 M $\Omega$ . The electrodes were tip-loaded with 50  $\mu$ M +/- ABA, backfilled with 300 mM KCl and connected by Ag/Ag half cells to the head stage (HS-2A x 0.01; Axon Instruments, Molecular Devices, Sunnyvale, CA, USA) of a TEVC amplifier (GeneClamp 500; Axon Instruments). Reference electrodes were prepared using capillaries filled with 300 mM KCl, sealed with an agar plug (2 % w/v agarose, 300 mM KCl and connected to ground with an Ag/Ag half-cell). Microelectrodes were

driven with a piezo-electric micromanipulator (MM3A; Kleindiek, Reutlingen, Germany). Prior to electro-infusion the electrode was brought in close proximity to an open stoma and left for 10 min to ensure no mechanical closing stimulus was applied. ABA or control solution was released from the capillary by a negative 10 min, 1 nA current-puls. After additional 10 min the image recording was stopped.

#### **ABA spray experiments**

*Arabidopsis* plants were ABA treated via spraying until leaves were moist. To investigate the dose dependency of the ABA receptor expression in wildtype plants, ABA solutions with 0 (control plants) 0.025, 0.1, 0.25, 2.5 and 25  $\mu$ M ABA in deionised water containing 1:10000 Triton-X100 were used. To investigate ABA regulated guard cell expression patterns, plants were sprayed with 25  $\mu$ M ABA solution or deionised water with Triton-X100. After 4h of incubation guard cells were sampled and RNA was extracted.

#### **CO<sub>2</sub> treatment for microarrays**

Single potted 6-7 week old *Arabidopsis thaliana* (Col 0) plants were subjected to an airstream containing ambient CO<sub>2</sub> (380ppm) and 50  $\pm$  5% RH (VPD = 1.322 kPa) in climate chambers at 22  $\pm$  2 °C and 100  $\mu$ mol m<sup>-2</sup> sec<sup>-1</sup> white light. After 1h half of the plants were treated with 1000 ppm CO<sub>2</sub> for 4h. Guard cells were sampled and RNA was extracted for microarray hybridisation.

#### **Darkness treatment for microarrays**

Single potted 6-7 weeks old *Arabidopsis thaliana* (Col 0) plants were subjected to an airstream containing ambient CO<sub>2</sub> (400ppm) and 50  $\pm$  5% RH (VPD = 1.322 kPa) in

climate chambers at  $22 \pm 2$  °C and 100  $\mu\text{mol m}^{-2} \text{sec}^{-1}$  white light. After 2 h half of the plants were kept into darkness for 2 h. Guard cells were sampled and RNA was extracted for microarray hybridisation.

#### **ABA and low RH (increased VPD) treatment**

These microarray data come from a previous study<sup>6</sup> in which the ABA and low humidity (increased VPD) signals persisted over 4 hours.

#### **Sampling and RNA Extraction**

Guard cell sampling and RNA extraction were performed as described<sup>6</sup>. Guard cell samples were frequently tested via vital staining to confirm that contaminations by other cell types remained below 5%–10%.

#### **Quantitative Real-Time PCR**

Quantitative real-time PCR (qPCR) was performed as described<sup>49</sup>. All transcripts were normalized to 10,000 molecules of actin2/8 and thus denoted as relative expression. All indicated replicate numbers refer to biological replicates. Primer sequences used: PYR1fwd 5'-GCTGACGAATTACAAATCCGTT-3', PYR1rev 5'-ACCGTCGCGAGTTTCTG-3', PYL1fwd 5'-CGTGAACGTGATAAGTGG-3', PYL1rev 5'-TGAACCGTCGTAACCGAT-3', PYL2fwd 5'-CATAACCCAACGCATCCA-3', PYL2rev 5'-AACTCAAGCCGCTCGGTA-3', PYL4 fwd 5'-CCGCTCGTTTTACACACAC-3', PYL2rev 5'-GTGTTGCCTGGAGGAACATC-3', PYL5fwd 5'-TGGTGCAGATGATCCACG-3', PYL4rev 5'-AGACTGAAGGTTGCACCG-3', PYL8fwd 5'-TGTGTGGTCACTTGTGAG-3', PYL8rev 5'-TGAACCGCAAGACGTTCA-3',

KCR2fwd 5'-ATGTGGATGCACTATCA-3', KCR2rev 5'-AAGGTTATCCGGTACAA-3',  
 ABI2fwd 5'-GGACTTAGAGGCTATTG-3', ABI2rev 5'-AGGATTAAATCCATTAGTG-3',  
 MYB60fwd 5'-ATGCTGTGACAAGATAGG-3', MYB60rev 5'-  
 AAAGTTTCCACGTTTAAT-3' CIPK25fwd 5'-AGATCCAAAACGTAGAAG-3',  
 CIPK25rev 5'-CTTACACAACCTCAACGAC-3',  
 HAI1fwd 5'-GTTGAATAGTTTTGACGA-3', HAI1rev 5'-GCCGTATTTAGGATAAGC-3',  
 ABRfwd 5'-GGTGGAATGATGGACAAG-3', ABRrev 5'-ATAAAGATCCAAATGGACG-  
 3', RAB18fwd 5'-AGAAGGGAATAACACAAA-3', RAB18rev 5'-  
 CAATACAACGACCGAA-3'.

## **PYL2 protein identification**

For the identification and quantification of PYL2, parallel reaction monitoring (PRM) via mass spectrometry was used. Four or five biological replicates from each experimental group were examined. Proteins were extracted from the ground GCs following the phenol extraction/ammonium acetate precipitation method described<sup>50</sup>. Briefly: 150 mg starting material was processed. For tryptic digestion, an aliquot of 20 µg protein was digested with 0.2 µg trypsin. Samples were cleaned using C18 solid phase extraction according to the manufacturer (Pierce™ C18 Spin Columns, Thermo Fisher Scientific, Gent, Belgium) and dissolved in 5% ACN, 0.1% formic acid. Peptides from the candidate proteins were designed using Skyline (version 4.2) and ordered from Thermo Fisher Scientific (UK) (PEPotec Grade 1). The ultra performance liquid chromatography - tandem mass spectrometer (UPLC–MS/MS) analysis was performed on an Ultimate 3000 UPLC system (Dionex, Thermo Scientific) equipped with a C18 PepMap100 precolumn (5 µm, 300 µm × 5 mm, Thermo Scientific) and an EasySpray C18 column (3 µm, 75 µm × 15 cm, Thermo Scientific)

using a gradient of 5% to 20% ACN in 0.1% formic acid (FA) for 10 min followed by a  
 gradient of 10% to 35% ACN in 0.1% FA for 4 min and a final gradient from 35% to  
 95% ACN in 0.1% FA for 2.5 min and a Q Exactive Orbitrap mass spectrometer  
 (Thermo Scientific, USA). The flow-rate was set at 250 µl/min. The Q Exactive was  
 operated in a positive ion mode with a nanospray voltage of 1.5 kV and a source  
 temperature of 250 °C. ProteoMass LTQ/FT-Hybrid ESI Pos. Mode CalMix  
 (MSCAL5-1EA SUPELCO, Sigma-Aldrich) was used as an external calibrant and the  
 lock mass 445.12003 as an internal calibrant. For the characterization of the  
 standard peptide library, the instrument was operated in a data-dependent  
 acquisition mode with a survey MS scan at a resolution of 70,000 (FWHM at m/z  
 200) for the mass range of m/z 350–1800 for precursor ions, followed by MS/MS  
 scans of the top 10 most intense peaks with + 2, + 3 and + 4 charged ions above a  
 threshold ion count of 16,000 at a 35,000 resolution using a normalized collision  
 energy (NCE) of 28 eV with an isolation window of 3.0 m/z and dynamic exclusion of  
 10 s. All data were acquired with Xcalibur 2.2 software (Thermo Scientific). The most  
 suitable peptide per protein was chosen to set the PRM analysis. To avoid  
 overlapping time windows the samples were analysed on the QE in PRM mode in 2  
 separate runs.

Peptide Name	Sequence	Peptide Length	m/z	Z	Start	End	Run
PYL2	LISGDGDVGSVR	12	587,8068	2	25,9	28,9	1

PRM analysis was performed in Skyline 4.2. The dicyano-1,4-benzoquinone (DDQ)  
 analysis of the peptide library was analysed with proteome discoverer and the library  
 was loaded in Skyline as a msf file. The NCBI Arabidopsis data file was loaded and



the results of the PRM run were loaded as raw files with the following settings: MS1 orbitrap detection 70 000 resolution MS2 orbitrap detection 20 000 resolution. Only peptides showing 3 accurate transitions were accepted.

### **Microarray Hybridization**

For transcriptome profiling samples were hybridized on an Agilent Platform using the Agilent *Arabidopsis* V4 (design number 021169) microarray chips (<http://www.agilent.com>). RNA quantity was measured with a ND-100 Spectrophotometer v3.3.0 (NanoDrop Technologies). RNA integrity was confirmed using an Agilent RNA 6000 Nano Chip on an Agilent 2100 BioAnalyzer (vB.02.03 BSI307). 500 ng total RNA were used for each sample labeling. Labeling and preparation of samples for hybridization was performed as described in the one-color microarray-based gene expression analysis protocol provided by Agilent including the one-color RNA spike-in kit (v5.0.1, 2006; Agilent Technologies, Santa Clara). Slides were scanned on the Agilent Microarray Scanner with extended dynamic range (XDR) at high resolution (5  $\mu$ m). Data sets were extracted by propriety software package (v9.5.3.1/ Agilent Technologies) using a standard protocol.

### **Array Analysis**

Data preprocessing was performed using the Bioconductor software<sup>51</sup> with the statistical programming environment R<sup>52</sup>. Normalization has been performed using negative control probes and quantile normalization using negative and positive control probes as implemented in the neqc function<sup>53</sup> of the Limma package<sup>54</sup>. Differential gene expression for all stimuli was calculated using the moderated t-statistic approach as implemented in the R-package Limma<sup>54</sup>, which has been specifically developed for the analysis of small sample size experiments. The p-

values of all results were corrected for multiple testing by using the false discovery rate (FDR)<sup>55</sup>. In order to identify guard cell specific gene regulation, we used a meta-analysis approach based on the order statistic of the stimulus p-values. Therefore, p-values from a one-sided moderated t-test of differential expressed genes between guard cells and leaf tissue have been derived from the Limma model. Subsequently, for each stimulus a combined p-value has been calculated using the second order statistics of the stimulus and the guard cell versus leaf p-value. This results in a set of genewise p-values for guard cell specific stimulus effects.

### **Pathway enrichment Analyses**

Analysis was carried out by a model-based approach which can directly be integrated into the Limma analysis of differential expression. A competitive gene set test has been applied to all stimulation contrasts as implemented in the function camera<sup>56</sup>. This function tests whether a set of genes is highly ranked relative to other genes in terms of differential expression, accounting for potential inter-gene correlation. Pathways for the analysis have been obtained from the MapMan catalogue<sup>57</sup> based on TAIR version 10 (2012), from which all gene sets with more than 5 genes and fewer than 500 genes have been included in the analysis.

### **Integrative Network Analysis**

Network data has been obtained from the STRING database (version 9.0, <http://string-db.org>)<sup>58</sup>. All interactions of the genome of *Arabidopsis thaliana* have been extracted from the database yielding a total of 717,810 interactions between 16,465 genes. For the analysis we used the high confidence network (combined score > 0.7) resulting in 69,603 interactions between 7,090 genes. After mapping all

genes on the array to the network the resulting largest connected component comprising 69,329 interactions between 6,724 genes has been extracted. This constitutes the base network used in all subsequent network analyses.

An optimal algorithm has been established to decompose the large intricate network structure into functionally coherent network modules<sup>59</sup>. These modules can be viewed as building blocks of cellular processes, such as basic metabolic pathways or stimuli-specific signalling. To identify these signalling sub networks the genes (network nodes) need to be scored according to their measured regulation and responsive modules can be identified using an exact search algorithm.

Here a statistical model to derive functional scores of closing signal responsiveness in guard cells has been used. Therefore, all stimuli p-values have been derived from the Limma analysis as detailed above. Integrated network analysis node (gene) scores have been computed based on these p-values as detailed in<sup>59</sup> using the routines implemented in the R-package BioNet<sup>60</sup>. Briefly, a Beta-uniform mixture model (BUM) has been fitted to the p-value distribution, thereby decomposing the signal and noise components of the distribution. Based on this, network scores have been calculated as log likelihood ratio of the signal to the noise component. Thus, positive scores reflect signal content (low p-values) whereas the negative scores reflect non-significant genes (noise). The signal to noise threshold has been multiple testing adjusted using the FDR. For the different stimuli appropriate FDR values have been selected according to the different effect strengths controlling the size of the resulting modules. These mainly contain either genes with a maximal response, but also a few tightly associated genes that show no differential regulation on the transcriptomic level (represented as squares in the network). Subsequently, an

exhaustive network search has been performed on the entire node-scored network to identify the maximum scoring sub networks using an exact algorithm<sup>59</sup>. The resulting optimal solutions represent maximally significant differentially regulated modules. Based on the functional scoring of stimulation this constitutes the optimal responsive modules within the entire network. This also means that no other module shows a stronger guard cell-specific closing signal response.

## **Statistics**

To test significances on qPCR (Supplementary Figs. 3, 6 and related Tables 1, 2) we used a multivariable linear model on log transformed expression values (relative to actin) adjusting additionally for day of experiment. Heteroscedasticity-consistent (HC) standard errors for regression coefficients have been calculated as implemented in sandwich package using default setting<sup>61</sup>. For dose response experiments (Supplementary Fig. 3) comparisons of different concentrations to the unstimulated control have been multiple testing corrected based on Dunnet's Post hoc test procedure for many-to-one comparisons. We used the step down adjustment (method="free") as implemented in the 'multcomp' package in R<sup>62</sup>. Reported p-values are based two-sided tests, p-values < 0.05 have been regarded as significant. Levels of significance were \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .

## **Data availability**

The data that support the findings of this study are available from the corresponding authors upon reasonable request. Microarray data of the ABA and low air humidity treatments which were taken from<sup>6</sup> were deposited in the Gene Expression Omnibus

(GEO) database <http://www.ncbi.nlm.nih.gov/geo> with accession no. GSE41054. The microarray data from CO<sub>2</sub> and darkness experiments were deposited at the same database under GSE118520.

#### **Code availability**

Algorithms and statistics used in the analyses are based on published approaches available in R packages (mainly Bioconductor framework) and other cited public available repositories.

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#### **Author contributions**

M.D. and T.M. conceived and conducted bioinformatics. H.M.M., H.B. and P.A. conceived, performed and analysed the expression studies. H.M.M. conducted and

analysed gas exchange measurements. M.P.L. and P.L.R. conceived and conducted the generation of transgenic plants. C.M.G. and S.C.C. conceived and conducted proteomic analyses. J.H. conceived, conducted and analysed electro infusion experiments. P.A., P.L.R., H.K., K.A.S.A.-R., T.M., A.M.H. and R.H. designed and conceived the study. M.D., T.M., P.A., A.H.M. and R.H. wrote the manuscript. All authors discussed the results and commented on the manuscript.

### **Competing interests**

The authors declare no competing financial interests.

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## Figure legends

**Fig. 1 Guard cell signalling pathway analysis.** MapMan pathways with a significant enrichment in at least one stimulus are visualized in a heatmap. Pathways are listed in rows and stimuli in columns. Darker shades (corresponding to the negative decadic logarithm of the enrichment pvalue) depict more significant enrichments. For each treatment and the respective controls four microarrays were analysed except darkness (n=3). All replicates represent biologically independent samples. A competitive pathway enrichment test was performed as implemented in the camera function from the limma package based on the gene expression data.

**Fig. 2 Integrative networks analysis identify distinct stomatal closing signal modules.** Stimulus specific modules: Optimal responsive sub networks of regulated genes in the entire network for a, ABA, b, RH, c, darkness, and d, CO<sub>2</sub> stimulation. Circles denote positive scoring genes (with highly significant expression changes)

911 whereas rectangles denote negative scoring nodes (e.g. additional nodes of modest  
 912 regulation) which are implicated by the algorithm on the bases of their functional  
 913 network context. Red indicates up regulation, green down regulation, where darker  
 914 colours correspond to higher fold change values. Gene clusters of common cellular  
 915 processes are enclosed by shaded lines highlighting the functional sub structure of  
 916 the modules. Only ABA-responsive genes are named (for details see Supplementary  
 917 Fig. 2). e, ABA receptors are main components in adaptation to stomatal closing  
 918 signals. Each module contains a cluster of typical ABA responsive genes. In all cases  
 919 ABA receptors are affected. Among the closing signals the kind of receptors as well  
 920 as the direction of regulation differ markedly indicating an altered sensitivity towards  
 921 ABA in the adaptation process to the individual signals. For each treatment and the  
 922 respective controls four microarrays were analysed except darkness (n=3). All  
 923 replicates represent biologically independent samples. The network analysis is based  
 924 on the statistics and p-values described in Table S1a.

925  
 926 **Fig. 3. PYL2 is sufficient for stomatal ABA sensitivity.** Excised leaves, in  
 927 darkness were preincubated with ABA fed via the petiole to prevent stomatal opening  
 928 in the light. Stomatal aperture was measured by gas exchange as a function of  
 929 relative transpiration. a, Stomata of wildtype and the 11458-mutant (PYL2 present)  
 930 remained almost closed, while those of the 12458-mutant appeared ABA-insensitive.  
 931 b, PYL2 in the 12458-background fully restored the wildtype phenotype, while PYR1  
 932 reacted with incomplete ABA-sensitivity. c, PYL4 and PYL5 were unable to restore  
 933 ABA sensitivity of the 12458-quintuple background line. Values are normalized to  
 934 timepoint 0. n=28 for Col0, n=6 for 12458, n=9 for 11458, n=5 for PYR1::12458, n=5

for PYL2::12458, n=6 for PYL4::12458, n=7 for PYL5::12458. All replicate numbers represent biologically independent samples, mean±SE.

**Fig. 4. ABA-induced movements of individual stoma.** Electro-infusion of ABA to single stoma. a, Stomata of wildtype, 11458-mutants (PYL2 present) and the PYL2::12458 complementation line closed within 10 minutes following an ABA-pulse. In contrast, stomata of the 12458-mutant (without PYL2) and the PYL5::12458 complementation line remained fully open. b, Amplitudes of the changes in stomatal pore width from fully open to maximum closed after ABA infusion revealed that PYL2 presence is sufficient to close stomata to wildtype levels. Stomata of the 12458 mutant remained open. In contrast to all other samples, the width of PYL5::12458 stomata showed little but ABA-independent closure over time. c, stomatal width of wildtype and mutants after 20 minutes illumination. Only 12458 mutants showed significantly wider stomatal pores prior to ABA application. n=9, all replicate numbers represent biologically independent samples, mean±SE.

**Fig. 5 PYL4 and 5 render guard cells CO<sub>2</sub>-sensitive.** Whole plants were placed in gas exchange cuvettes in the dark at 400 ppm CO<sub>2</sub> and stomatal aperture was measured as a function of the relative transpiration by gas exchange. Following stomatal opening in the light the CO<sub>2</sub> concentration was increased to 1000 ppm. a, Stomata of wildtype plants closed about 60%, while both quintuple-mutants did not react. b, The PYL2 complemented 12458-mutant did not react to elevated CO<sub>2</sub> and also the PYR1 complemented stomata remained impaired. c, 12458-mutants complemented with either PYL4 or PYL5 closed their stomata in a wildtype manner. Values are normalized to timepoint 0. n=20 for Col0, n=12 for 12458, n=6 for 11458,

n=4 for PYR1::12458, n=6 for PYL2::12458 and n=7 for PYL5::12458. All replicate numbers represent biologically independent samples, mean  $\pm$  SE.

PYR1	At4g17870					64
PYL1	At5g46790					
PYL2	At2g26040				39	18
PYL4	At2g38310			66	36	19
PYL5	At5g05440				33	16
PYL8	At5g53160				77	54
MYB60	At1g08810					42
ABR	At3g02480				1331	5679
HAI1	At5g59220		173*		594	1535
ABA		25 nM	100 nM	250 nM	2,5 $\mu$ M	25 $\mu$ M

**Table 1 ABA-dose-response of the transcription of guard cell ABA-receptor and ABA-regulated genes.** Upon ABA-spray application transcription was either induced, reduced or remained unchanged. Numbers represent the expression changes presented in Supplementary Fig.3 (in percent) relative to untreated control. Blue = down-regulation, yellow to red = up-regulation, grey = no change in expression. Asterisk denotes that this value has weak significance and represents only 11 % of the maximum induction (for sample sizes and statistics see related Supplementary Fig. 3 and Methods).

	MYB60	ABI2	CIPK25	KCR2	RAB18
wt	26	268	268	195	400
12458 (PYL1 present)		298	269	285	1218
PYR1::12458	40		303	194	1436
PYL2::12458	38	251	228	200	731
PYL4::12458		307	331	244	491
PYL5::12458		314			381
11458 (PYL2 present)	31				649

PYR1::11458	22	203			956
PYL1::11458	14	160	178		433
PYL4::11458	25				421
PYL5::11458	25				301
PYL8::11458	43		215		493

**Table 2 Individual ABA receptor family members control the expression of downstream genes.** ABA-spraying (25  $\mu$ M) led to up or downregulation of genes in wildtype plants. Quintuple knockout and complementation lines revealed the receptors necessary for ABA-induced gene-regulation. Numbers represent the expression changes presented in Supplementary Fig.6 (in percent) relative to untreated control. Blue = downregulation, yellow to red = up-regulation, grey = no change in expression. (for sample sizes and statistics see related Supplementary Fig. 6 and Methods).











